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Potential Involvement of Ferroptosis in Duchenne Muscular Dystrophy-Associated Cardiomyopathy

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Abstract

Background/Objectives: Cardiomyopathy (CM) is a leading cause of morbidity and mortality in Duchenne muscular dystrophy (DMD) patients. Ferroptosis, an iron-dependent form of cell death characterized by lipid peroxidation, is implicated in various cardiovascular diseases. However, the role of ferroptosis in DMD-CM remains unexplored. **Methods:** Here, we used dystrophin and utrophin double-knockout (*mdx:utr*^{−/−}) mice as a model that exhibits cardiac pathological phenotypes similar to those seen in DMD patients to investigate the potential role of ferroptosis. **Results:** We observed an increased level of iron deposition and lipid peroxidation in the hearts of *mdx:utr*^{−/−} mice. Live/Dead viability assays revealed that *mdx:utr*^{−/−} cardiomyocytes exhibited greater susceptibility to ferroptosis than WT cardiomyocytes both at baseline and upon exposure to ferroptosis inducers. We also used *mdx:utr*^{−/−} mice with a heterozygous sarcolipin (SLN) knockout background (*sln*^{+/-}) to investigate the effect of SLN reduction on ferroptosis susceptibility in DMD-CM. Notably, ferroptosis was significantly suppressed in cardiomyocytes from *mdx:utr*^{−/−}:*sln*^{+/-} mice ($p < 0.01$). Western blot analysis confirmed the upregulation of transferrin receptor 1 (TfR1) and 15-lipoxygenase-1 (15LOX1), along with the downregulation of heme oxygenase-1 (HMOX-1) and ferroptosis suppressor protein 1 (FSP1) in *mdx:utr*^{−/−} hearts, while glutathione peroxidase 4 (GPX4) levels remained unchanged. A similar pattern of alterations in ferroptosis-related biomarkers was observed in human heart samples from DMD patients compared to healthy controls. **Conclusions:** Our results provide direct evidence that ferroptosis contributes to the pathology of DMD-CM and suggest that reducing SLN expression and inhibiting ferroptosis may represent potential therapeutic strategies for this condition.

Keywords: Duchenne muscular dystrophy; cardiomyopathy; ferroptosis; ferroptosis-related biomarkers



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1. Introduction

Duchenne muscular dystrophy (DMD) is the most common and fatal form of muscular dystrophy that is caused by loss-of-function mutations in the dystrophin gene. By adulthood, most DMD patients develop cardiomyopathy, which remains the leading cause of death in these patients [1]. Despite extensive research, the mechanism(s) underlying DMD-associated cardiomyopathy (DMD-CM) remain poorly understood. As a result, there is no effective treatment for DMD-CM available. Recent studies, including our group, have suggested that ion channel remodeling (e.g., Cx43 alterations) [2–5], fibrosis [6],

microtubule disorganization [7], calcium dysregulation [8,9], and mitochondrial dysfunction [8,10] may contribute to the pathogenesis of DMD-CM. It has also been shown that in dystrophin/utrophin double knockout (*mdx:utr*^{−/−}) mouse models of DMD, there is an up-regulation of sarcolipin (SLN), a known inhibitor of sarco/endoplasmic reticulum (SR) Ca²⁺ ATPase (SERCA), and that this likely plays a substantial role in the pathogenesis of DMD-CM. Conversely, reducing SLN expression in *mdx:utr*^{−/−} mice rescued cardiac function by improving Ca²⁺ cycling and mitochondrial function [8,9]. Additionally, multiple forms of cell death, including apoptosis and necroptosis, have been implicated as significantly contributing to and playing a central role in the pathogenesis of various cardiovascular diseases, e.g., myocardial infarction and heart failure [11,12] as well as DMD-CM [13–17]. However, it remains unclear whether ferroptosis, a recently identified form of regulated cell death, contributes to the progression of DMD-CM.

Ferroptosis is a novel regulated nonapoptotic cell death characterized by iron dependence and the accumulation of lipid peroxidation that results in cell and/or mitochondrial membrane damage [18–21]. The original study by Dixon et al. [18] has shown that ferroptosis is driven by the depletion of glutathione peroxidase 4 (GPX4), an enzyme that protects cells from oxidative damage. The iron accumulation triggers a cascade of events, including the generation of reactive oxygen species (ROS) and lipid peroxidation, ultimately leading to cell damage and death. We and others have shown that direct iron treatment or cardiac iron overload induces ferroptosis in cardiomyocytes, consequently leading to cardiac dysfunction [22,23]. More studies have revealed that multiple signaling pathways (e.g., FSP1-CoQ 10-NADH and GSH-GPX4 axis) and associated biomarkers are essential for regulating ferroptosis. These ferroptosis-related biomarkers include transferrin receptor (TfR1), heme oxygenase-1 (HMOX1), and fatty acid-CoA ligase 4 (FACL4), among others. [19,24–26]. While there is not a specific assay for ferroptosis detection at the tissue level, the occurrence of ferroptosis can be determined through a combined assessment of iron and ROS dependency, lipid peroxidation, expression of ferroptosis-related biomarkers, and inhibition by the specific inhibitor ferrostatin-1 (Fer-1) [27–29].

Ferroptosis is currently thought to suppress tumor growth and enhance the sensitivity of various cancers to chemotherapy and immunotherapy. On the other hand, it can also induce damage to normal tissues and organs and contribute to the onset and progression of diseases such as neurodegenerative diseases (e.g., Alzheimer's and Parkinson's disease) and musculoskeletal diseases [30], as well as cardiovascular diseases [19,31,32]. Emerging evidence suggests that ferroptosis plays an essential role in the development of iron overload cardiomyopathy, doxorubicin-induced cardiotoxicity, ischemia/reperfusion (I/R) injury, heart failure (HF), and atherosclerosis.

In the context of DMD or DMD-CM, it has been reported that the blood iron level is higher in dystrophic mouse strains [33] and that iron deprivation may exert therapeutic effects [34]. It has also been demonstrated that HMOX1 is blunted in the muscles from the *mdx* mouse model of DMD and that overexpression of HMOX1 reverses the DMD phenotype [35]. In addition, a recent study using dystrophin-deficient human iPSC-derived cardiomyocytes (hiPSC-CMs) has demonstrated dysregulated iron homeostasis in these cells [36]. Furthermore, idebenone, a synthetic derivative of the known antioxidant CoQ10 and ferroptosis inhibitor, has been shown to be cardioprotective in DMD-CM [37]. Collectively, these previous findings strongly suggest that ferroptosis may be a potential contributor to DMD-CM and provide a clear rationale for the present study. Progressive loss of functional cardiomyocytes due to ferroptosis and the resulting cardiac remodeling may lead to cardiac dysfunction or heart failure in DMD patients.

Here, we aim to investigate the potential role of ferroptosis in the pathogenesis of DMD-CM using the *mdx:utr*^{−/−} mouse model, which displays a cardiomyopathic phenotype. We

have demonstrated for the first time that *mdx:utr*^{−/−} cardiomyocytes are more susceptible to ferroptosis and that lowering SLN expression reduces this vulnerability. This study highlights a potential therapeutic avenue involving SLN reduction to prevent ferroptosis-related cardiac complications in DMD.

2. Methods and Materials

2.1. Mouse Models

We used dystrophin and utrophin double-knockout (*mdx:utr*^{−/−}) mice as a model of severe muscular dystrophy. Earlier studies have indicated that it recapitulates the clinical features of both skeletal muscular dystrophy and cardiomyopathy. We also used these double-knockout mice in a sarcolipin (SLN) heterozygous-knockout background (*mdx:utr*^{−/−}:*sln*^{+/-}) [8,9]. The susceptibility to ferroptosis was evaluated in *mdx:utr*^{−/−} cardiomyocytes vs. WT cardiomyocytes, as well as the potential effect of SLN in cardiomyocytes from *mdx:utr*^{−/−}:*sln*^{+/-} vs. WT. Both sexes of mice aged 2–4 months were used. Care and use of animals in all experiments were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Rutgers University-New Jersey Medical School. All procedures conformed to the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Lipid Peroxidation—Malondialdehyde (MDA) Assay

Malondialdehyde (MDA), a byproduct of lipid peroxidation, is an indicator of membrane lipid peroxidation and was used to assess membrane damage and ferroptosis. We conducted the MDA assay (DTBA-100, BioAssay Systems, Hayward, CA, USA), which involves reacting MDA with thiobarbituric acid (TBA) to form a colored adduct. This adduct, which exhibits a red-pink color, can then be measured spectrophotometrically at 532 nm, allowing for the determination of MDA concentration. Sample preparation, assay, and measurements were conducted following the company's protocol [<https://bioassaysys.com/wp-content/uploads/DTBA.pdf> (accessed on 13 February 2026)].

2.3. Isolation of Mouse Left Ventricular Cardiomyocytes

Left ventricular (LV) cardiomyocytes were enzymatically isolated from the LV free wall of mouse hearts as described previously [38,39]. Briefly, the hearts were removed from isoflurane-anesthetized mice. The hearts then underwent retrograde perfusion at 37 °C in Langendorff fashion with nominally Ca²⁺-free Tyrode's solution containing 0.5 mg/mL collagenase (Type II; Worthington Biochemical Co., Lakewood, NJ, USA) and 0.1 mg/mL thermolysin (Sigma-Aldrich, St. Louis, MO, USA) for 10–12 min. The enzyme solution was then washed out, and the hearts were removed from the perfusion apparatus. The LV was removed and placed in a Petri dish. LV cardiomyocytes were isolated after being teased apart using forceps and filtered through a nylon mesh. The Ca²⁺ concentration was then gradually increased to 1.0 mM, and the cells were stored at room temperature. Ventricular cardiomyocytes were cultured for ~18 h for Live/Dead cell viability assays.

2.4. Determination of Ferroptosis Using Live/Dead Cell Viability Assay

We conducted Live/Dead cell viability assays in cultured ventricular cardiomyocytes isolated from the hearts of WT, *mdx:utr*^{−/−}, and *mdx:utr*^{−/−}:*sln*^{+/-} mice as previously described [22]. Ventricular cardiomyocytes were first incubated on laminin-coated culture dishes in a plating medium (Medium 199, 5% FBS, 25 µM blebbistatin, 1% Pen Strep, 1% Glutamax) for 2 h and then cultured in a culture medium (Medium 199, BSA 100 µg/mL, 25 µM blebbistatin, ITS 10 µg/mL, 1% Pen Strep, 1% Glutamax) for ~18 h. Ventricular cardiomyocytes were cultured under control conditions or treated with various ferroptosis inducers. Cardiomyocytes were then stained with 2 µM calcein AM and 4 µM ethidium

homodimer-1 (EthD-1) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 37 °C. Live cells were stained with calcein AM and visualized as green (Ex/Em: 485/530 nm), while the nuclei of dead cells were stained with EthD-1 and visualized as red (Ex/Em: 530/617 nm). Cell death rate was quantified as the ratio of dead cells to the total number of cells in three randomly selected fields per culture dish ($n = 3\text{--}7$ dishes from 2 to 4 mice in each group).

2.5. Histological Examination of Iron Deposition in the Heart

Left ventricle free wall samples were excised from mouse hearts, rinsed with phosphate-buffered saline (PBS), and fixed in 10% neutral formalin. The specimens were embedded in paraffin and sectioned ($\sim 5\text{ }\mu\text{m}$). Perls' Prussian blue was used to determine iron deposition. Perls' Prussian blue stain evaluates the total accumulation of ferric iron (Fe^{3+}) in ventricular tissue, which should include cardiomyocytes, non-myocytes (e.g., fibroblasts, immune cells, vascular cells), and iron in the extracellular matrix (e.g., collagen and fibronectin).

2.6. Human Samples

Two non-DMD and two DMD male human ventricular samples [9,40] were obtained from the University of Maryland Brain and Tissue Bank, a member of the NIH Neuro-BioBank network. All samples were dissected post-mortem. The cause of death in DMD1 was cardiac failure at age 15, while in DMD2 it was pulmonary thromboembolism at age 17. The research use of these samples was approved by the Institutional Review Board (IRB) at Rutgers New Jersey Medical School (Pro20140000334, exempt). The study was conducted in accordance with the principles of the Declaration of Helsinki.

2.7. Western Blot Analysis

Ventricular tissues from WT, *mdx:utr*^{−/−} and *mdx:utr*^{−/−}:*sln*^{+/-} mice and human samples were used for Western blot analysis. The tissues were dissected out, rinsed in sterile PBS, and flash-frozen in liquid nitrogen. Tissue homogenization was performed in lysis buffer supplemented with protease and phosphatase inhibitors. The lysates were mixed with 2× loading buffer (Laemmli Sample Buffer, #1610737, Bio-Rad, Hercules, CA, USA) and 5% 2-mercaptoethanol (#1610710, Bio-Rad) and heated at 95 °C for 5 min. Equal amounts (20–30 μg) of total protein extracts along with pre-stained molecular weight markers were separated by SDS-PAGE and transferred onto PVDF membranes. After transferring, the membranes were stained with Ponceau S and cut into small strips according to the molecular weight of each protein being studied. The membrane strips were then blocked with 3% milk in Tris Buffered Saline with Tween 20 (TBST) and probed overnight at 4 °C or for 2 h at room temperature using antibodies specific for 15LOX1 (1:1000, ab244205, Abcam, Cambridge, MA, USA); Hmox-1 (1:2000, ab13243, Abcam); FSP-1 (1:1000, PA5-103183, Invitrogen, Thermo Fisher Scientific); NOX-4 (1:1.000, NB110-58849, Novus Biologicals, Centennial, CO, USA); GPX4 (1:2000, ab125066, Abcam); FAC14 (1:1000, ab205199, Abcam); TfR1 (1:500, AB214039, Abcam); GCH1 (1:1000, A10616, ABclonal, Woburn, MA, USA); COX2 (1:500, ab179800, Abcam); GAPDH (1:7000, AC002, ABclonal). Membranes were incubated with appropriate secondary antibodies for 1 h at room temperature and visualized using SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) on a Bio-Rad ChemiDoc MP Imaging system. Quantitation of signals was performed using Image Lab version 5.1 software and then normalized to GAPDH levels.

2.8. Statistical Analysis

Results are expressed as mean $\pm$ standard error of mean (SEM). OriginPro software version 2024b (OriginLab, Northampton, MA, USA) was used for data analysis. Sample/Animal numbers were determined based on effect sizes and variability observed in our previous studies using the same mouse model and experimental endpoints. These data indicated that the selected group sizes provide adequate power to detect biologically meaningful differences while minimizing animal use in accordance with the principle of reduction. Tests for normality and homogeneity of variance were performed prior to statistical analyses. Student's *t*-test and ANOVA followed by Tukey post hoc test were used for statistical analyses. Results were considered significantly different if the *p*-value was ≤ 0.05 .

3. Results

3.1. Alteration of Ferroptosis-Related Features: Iron Deposition and Membrane Lipid Peroxidation in *mdx:utr*^{−/−} Mouse Left Ventricles

The primary objective of this study was to investigate the potential involvement of ferroptosis in the pathogenesis of DMD-CM. Given that ferroptosis is characterized by an iron-dependent mechanism and the accumulation of membrane lipid peroxides, we first examined the iron deposition level within left ventricular (LV) tissue in *mdx:utr*^{−/−} and WT mice using Perl's Prussian Blue staining. We found significantly higher iron deposition in the LV tissue from *mdx:utr*^{−/−} mice ($0.610 \pm 0.162\%$ area) compared to that of WT controls ($0.031 \pm 0.006\%$ area) ($p < 0.05$) (Figure 1A). We further detected a statistically significant elevation of lipid peroxidation in *mdx:utr*^{−/−} hearts compared to WT ($p = 0.05$) as evidenced by the increased level of MDA, which is a byproduct of membrane lipid peroxidation (Figure 1B). These results suggest that the heart of DMD mice provides a milieu that facilitates the incidence of ferroptosis, which may partially contribute to the pathological mechanisms underlying DMD-CM.

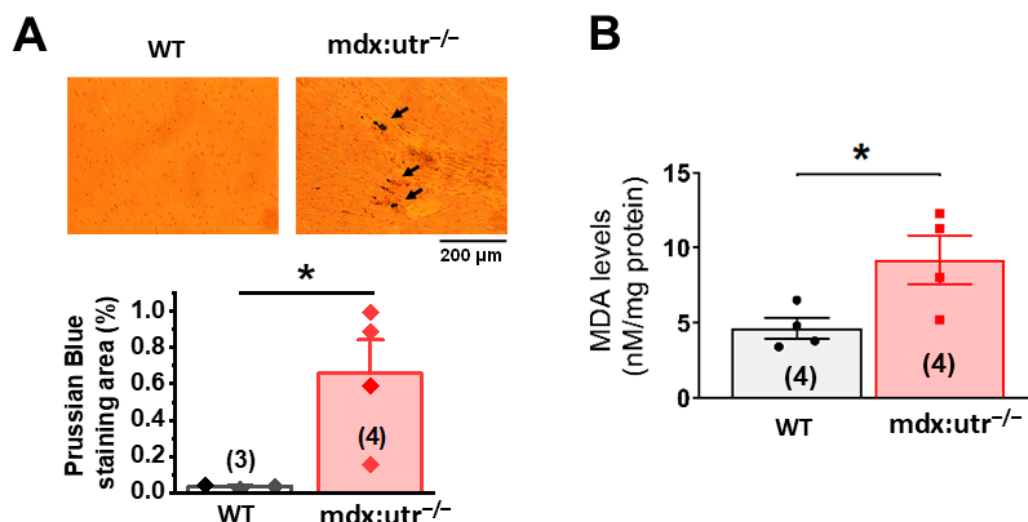


Figure 1. Increase Iron Deposition and Membrane Lipid Peroxidation in *mdx:utr*^{−/−} Mouse Hearts. (A): Representative Prussian blue staining and quantitative analysis of iron deposition in the left ventricles. Iron deposition spots are indicated by arrows in the *mdx:utr*^{−/−} panel. Magnification: 20×10 . (B): The level of Malondialdehyde (MDA), an indicator of membrane lipid peroxidation, in *mdx:utr*^{−/−} vs. WT mouse left ventricular heart tissue. * $p \leq 0.05$.

3.2. Susceptibility to Ferroptosis: Enhancement in *mdx:utr*^{−/−} and Attenuation in *mdx:utr*^{−/−}:*sln*^{+/-} Cardiomyocytes

Next, we evaluated the ferroptosis susceptibility of *mdx:utr*^{−/−} mouse cardiomyocytes in comparison to WT. As shown in Figure 2A,B, we carried out Live/Dead viability assays in ventricular cardiomyocytes from WT (left panel) and *mdx:utr*^{−/−} (middle panel) mice under basal conditions (i.e., in the absence of any ferroptosis inducers). We found a significantly higher level of ferroptosis in *mdx:utr*^{−/−} cardiomyocytes than in WT cardiomyocytes. The death rate was 42.1 ± 8.4% in *mdx:utr*^{−/−} cells compared to 8.7 ± 1.5% in WT cells ($p < 0.01$). The role of ferroptosis was further confirmed using the specific ferroptosis inhibitor Fer-1, a radical-trapping antioxidant (RTA) that targets lipid peroxidation. Treatment with 10 μM Fer-1 almost completely prevented the cell death in *mdx:utr*^{−/−} cardiomyocytes, with the death rate being markedly decreased to 7.4 ± 1.9% (Figure 2A,B). In addition, we also found that cell death in *mdx:utr*^{−/−} cardiomyocytes showed a strong tendency to be prevented by the iron chelator deferoxamine (DFO), but not by the apoptosis blocker emricasan (EMR), consistent with its iron dependency and ferroptotic nature (Supplement Figure S1).

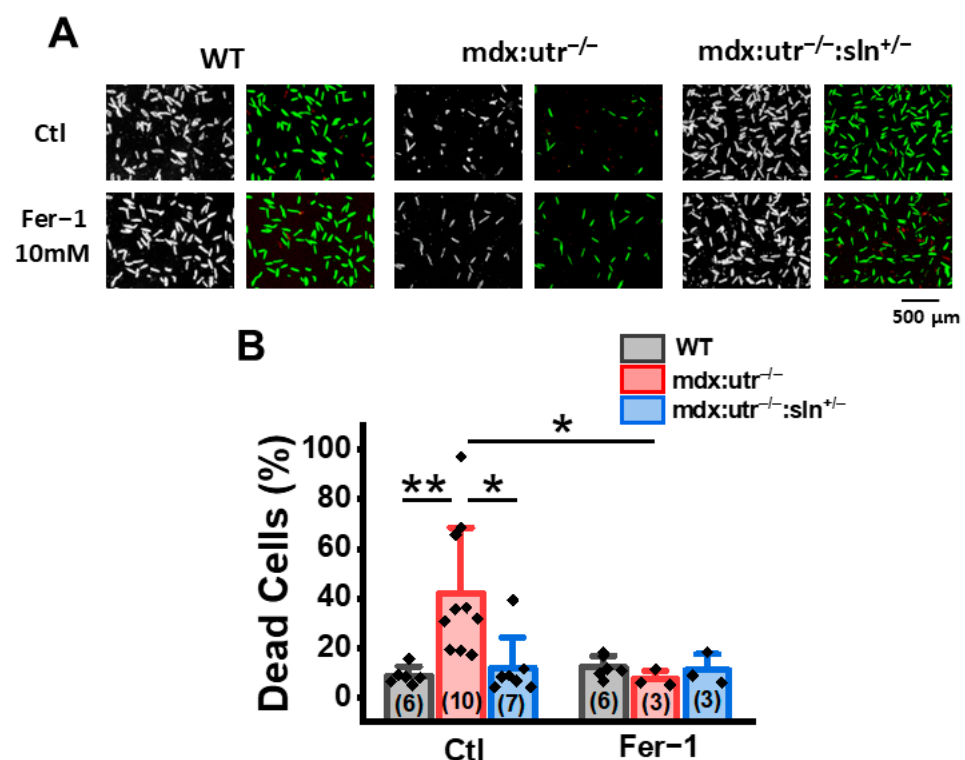


Figure 2. Increased ferroptosis susceptibility in *mdx:utr*^{−/−} cardiomyocytes and its mitigation by SLN downregulation (*mdx:utr*^{−/−}:*sln*^{+/-}) under basal conditions. (A): Representative images of WT, *mdx:utr*^{−/−}, and *mdx:utr*^{−/−}:*sln*^{+/-} cardiomyocytes cultured for ~18 h under basal conditions or treated with 10 μM Fer-1. Brightfield (left) and Live/Dead (right) images are shown for each condition. Live cells were stained with calcein AM (green), and nuclei of dead cells were stained with ethidium homodimer-1 (red). (B): Quantitation of the cell death rates, which were calculated as dead cells/total cells. * $p < 0.05$ and ** $p < 0.01$, as determined by two-way ANOVA followed by Tukey's post hoc test.

Our previous study has demonstrated that reducing SLN expression ameliorates dystrophic pathology and dysfunction in DMD-CM [9]. The underlying mechanism may involve an improvement in Ca²⁺ handling and mitochondrial function [8,9]. Here, we investigated the effect of SLN reduction on ferroptosis susceptibility in our DMD-

CM model. As shown in Figure 2A,B (right panel), the basal cell death rate was significantly lower in *mdx:utr*^{-/-}:*sln*^{+/-} cardiomyocytes ($11.8 \pm 4.7\%$) compared to that in *mdx:utr*^{-/-} cardiomyocytes.

Similarly, Live/Dead viability assays also showed that *mdx:utr*^{-/-} cardiomyocytes were more susceptible to ferroptosis exacerbated by ferroptosis inducers such as the bioavailable form of iron salt ferric ammonium citrate (FAC) and the GPX4 inhibitor RSL3. As shown in Figure 3A,B, 2 mM FAC induced a higher death rate in *mdx:utr*^{-/-} cells ($98.0 \pm 0.7\%$, middle panel) than in WT ($52.9 \pm 8.4\%$, left panel) ($p < 0.01$), which were both suppressed to the same extent ($23.1 \pm 5.0\%$ and $21.1 \pm 5.7\%$ $p = 0.83$) in *mdx:utr*^{-/-} and WT cells, respectively) by 10 μ M Fer-1. Additionally, the effect of reducing SLN expression on susceptibility to ferroptosis was also evaluated. The death rate induced by FAC was $25.3 \pm 5.3\%$ in *mdx:utr*^{-/-}:*sln*^{+/-} cardiomyocytes, which was significantly reduced compared to *mdx:utr*^{-/-} cardiomyocytes (Figure 3A,B, right panel).

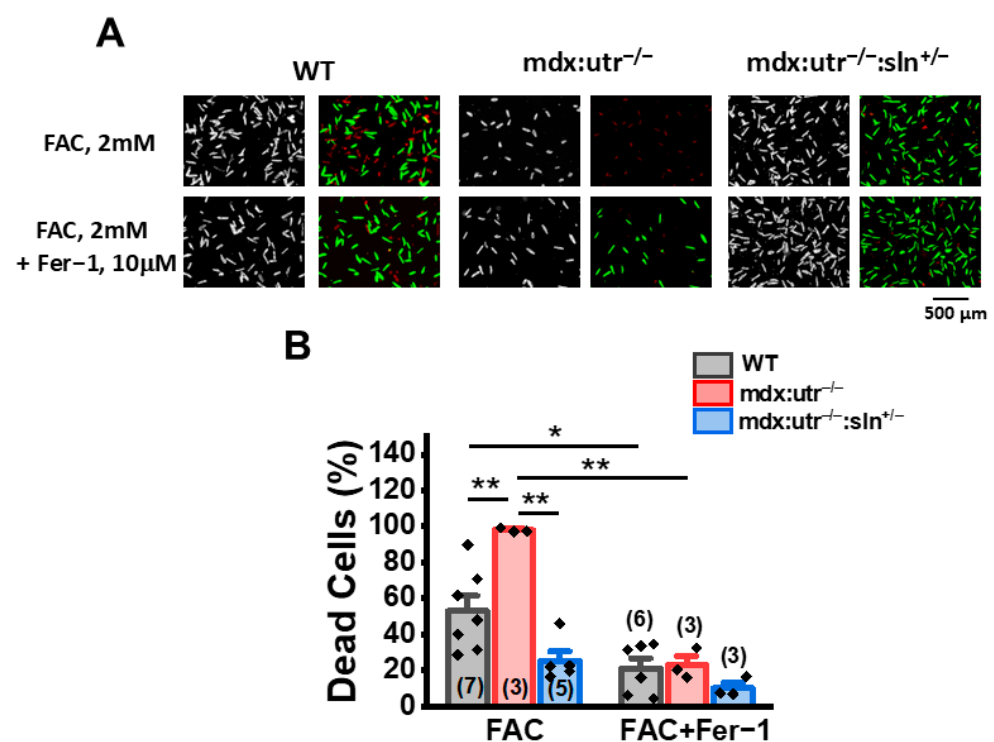


Figure 3. Increased susceptibility to ferroptosis induced by FAC in *mdx:utr*^{-/-} cardiomyocytes and its mitigation by SLN downregulation (*mdx:utr*^{-/-}:*sln*^{+/-}). (A): Representative images of WT, *mdx:utr*^{-/-}, and *mdx:utr*^{-/-}:*sln*^{+/-} cardiomyocytes cultured for ~18 h with 2 mM FAC or 2 mM FAC + 10 μ M Fer-1 treatment. (B): Quantitation of the cell death rates, which were calculated as dead cells/total cells. * $p < 0.05$ and ** $p < 0.01$, as determined by two-way ANOVA followed by Tukey's post hoc test.

Furthermore, as shown in Figure 4A,B, we also observed a significant increase in ferroptosis rates following RSL3 (0.5 μ M) treatment in both WT cells ($93.2 \pm 1.8\%$) and *mdx:utr*^{-/-} cells ($99.9 \pm 0.1\%$), with the rate in *mdx:utr*^{-/-} myocytes (middle panel) being marginally higher than that in WT myocytes (left panel). Meanwhile, the RSL3-induced ferroptosis rate was $91.2 \pm 3.7\%$ (Figure 4A,B, right panel) in *mdx:utr*^{-/-}:*sln*^{+/-} cardiomyocytes, which is also lower compared to that in *mdx:utr*^{-/-} cardiomyocytes. More interestingly, the sensitivity to Fer-1 (10 μ M) inhibition was extensively attenuated in *mdx:utr*^{-/-} cardiomyocytes. Fer-1 (10 μ M) markedly decreased the RSL3-induced ferroptosis rates in WT cardiomyocytes (to $35.8 \pm 7.2\%$), but failed to do so in *mdx:utr*^{-/-} cardiomyocytes ($98.1 \pm 1.9\%$). On the contrary, SLN reduction (*mdx:utr*^{-/-}:*sln*^{+/-} cardiomyocytes) potenti-

ated the sensitivity to Fer-1 inhibition on FAC- or RSL3-induced ferroptosis. For example, in the case of RSL3-induced ferroptosis (Figure 4A,B), 10 μ M Fer-1 markedly reduced the death rate to $6.4 \pm 1.7\%$ in *mdx:utr^{-/-}:sln^{+/-}* myocytes, even lower than that in WT cardiomyocytes ($35.8 \pm 7.2\%$).

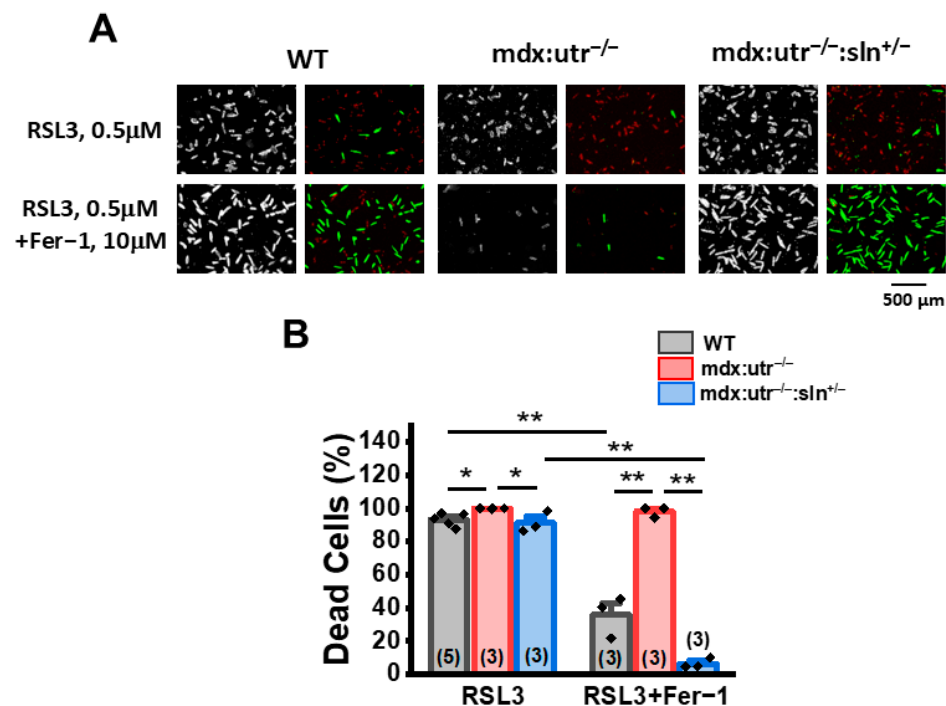


Figure 4. Increased susceptibility to ferroptosis induced by RSL3 in *mdx:utr^{-/-}* cardiomyocytes and its mitigation by SLN downregulation (*mdx:utr^{-/-}:sln^{+/-}*). (A): Representative images of WT, *mdx:utr^{-/-}*, and *mdx:utr^{-/-}:sln^{+/-}* cardiomyocytes cultured for ~18 h with 0.5 μ M RSL3 or 0.5 μ M RSL3 + 10 μ M Fer-1. (B): Quantitation of the cell death rates, which were calculated as dead cells/total cells. * $p < 0.05$ and ** $p < 0.01$, as determined by two-way ANOVA followed by Tukey's post hoc test.

These results indicate that *mdx:utr^{-/-}* cardiomyocytes are more susceptible to ferroptosis both at baseline and upon exposure to ferroptosis inducers, thereby supporting the notion that ferroptosis likely contributes to DMD-CM. Reducing SLN expression (*mdx:utr^{-/-}:sln^{+/-}*) alleviates the enhanced propensity for ferroptosis observed in *mdx:utr^{-/-}* cardiomyocytes and might be cardioprotective.

3.3. Alterations of Representative Ferroptosis-Related Biomarker Expression in *mdx:utr^{-/-}* Mouse Hearts

Ferroptosis is driven by iron-dependent lipid peroxidation and is regulated by numerous promotion and suppression pathways. Ferroptosis involvement can be determined based on its key features that include iron- and ROS-dependency, lipid peroxidation, GPX4 involvement, and responsiveness to specific ferroptosis inhibitors (e.g., Fer-1) [27–29]. Although a specific assay for detecting ferroptosis at the tissue level (analogous to TUNEL staining for apoptosis) is not yet available [28], several key molecules within these pathways have been considered as promising ferroptosis-related biomarkers [41,42]. Most ferroptosis-related biomarkers are relatively specific for detecting ferroptotic cell death. However, it should be noted that pathological features characteristic of DMD, such as Ca^{2+} overload, oxidative stress, and mitochondrial dysfunction, may also create a permissive environment for other forms of regulated cell death, including necroptosis and apoptosis. Therefore, the current experimental approaches were designed to examine the potential

role of ferroptosis, while acknowledging that other forms of regulated cell death may also contribute.

To identify potential alterations in ferroptosis-related biomarkers in *mdx:utr*^{−/−} mouse hearts, we subsequently performed Western blot analyses. As shown in Figure 5A,B, transferrin receptor (TfR1), 15-lipoxygenase-1 (15LOX1), and cyclooxygenase-2 (COX2) were markedly upregulated, with fatty acid-CoA ligase 4 (FACL4) showing a trend toward increased expression in *mdx:utr*^{−/−} mouse heart tissue. However, no changes were observed in the expression of GTP cyclohydrolase 1 (GCH1) or glutathione peroxidase 4 (GPX4). In contrast, ferroptosis suppressor protein 1 (FSP1), heme oxygenase 1 (HMOX1), and NADPH oxidase 4 (NOX4) were downregulated in *mdx:utr*^{−/−} heart tissue. Despite certain discrepancies, the Western blotting results collectively support an enhanced propensity for ferroptosis in *mdx:utr*^{−/−} cardiomyocytes, which likely contributes, at least in part, to the pathological mechanisms of DMD-CM. It should be noted that SLN reduction (*mdx:utr*^{−/−}:*sln*^{+/-} heart tissue) did not reverse the altered expression levels of these ferroptosis biomarkers, although a trend was observed in some, such as 15LOX1.

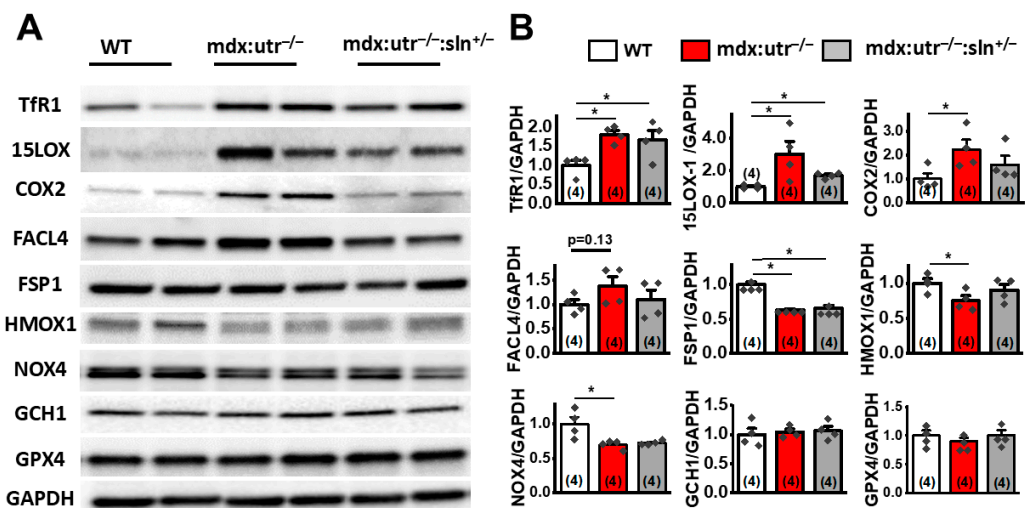


Figure 5. Alterations in the expression of representative ferroptosis-related molecules in *mdx:utr*^{−/−} and *mdx:utr*^{−/−}:*sln*^{+/-} mouse hearts. (A): Representative Western blot bands of various ferroptosis-related molecules. (B): Quantification of ferroptosis-related molecule expression normalized to GAPDH loading control. Data were normalized to WT on the same gel ($n = 4$ mice in each group). * $p < 0.05$, as determined by one-way ANOVA.

3.4. Assessment of Ferroptosis-Related Biomarkers in Heart Tissues of Human DMD Patients

Next, we performed Western blot analysis of several ferroptosis-related biomarkers in human ventricular biopsies and compared them with those from non-DMD controls. As shown in Supplement Figure S2, we observed a marked tendency toward increased expression of TFRC, 15LOX1, and FACL4 along with decreased expression of NOX4 in DMD heart tissues, while GPX4 expression level showed minimal alteration. These results were consistent with the changes observed in *mdx:utr*^{−/−} mouse heart tissue. In contrast, FSP1 expression also showed a tendency to increase. These findings support clinical relevance, although no statistical significance was detected due to the small sample size ($n = 2$ subjects per group).

4. Discussion

4.1. Potential Contribution of Ferroptosis to DMD-CM Pathology and Alleviation of Ferroptosis by SLN Reduction

DMD is an X-linked genetic disorder caused by mutations in the DMD gene, which encodes dystrophin. DMD-CM is a major cause of morbidity and mortality in DMD patients [1]. Nevertheless, the pathophysiological mechanisms underlying DMD-CM remain inadequately elucidated, and no effective therapeutic strategies are currently available. It has been suggested that the lack of dystrophin makes cardiomyocytes more susceptible to damage and programmed cell death, contributing to progressive cardiomyopathy and death in DMD patients. Previous studies have demonstrated that different forms of cell death, including apoptosis, necroptosis, ferroptosis, and pyroptosis, play a central role in various cardiovascular diseases, such as myocardial infarction and heart failure, by contributing to loss of cardiomyocyte function, cardiomyocyte cell death, and subsequent heart damage [11,12]. Ferroptosis, a newly recognized type of regulated cell death characterized by iron dependence and the accumulation of cell membrane lipid peroxidation, has also been shown to be involved in various cardiovascular disease conditions [19,31]. Although recent studies revealed iron dysregulation, oxidative stress, and Ca^{2+} mishandling in DMD, it remains unclear whether ferroptosis contributes to DMD-CM. In the present study, we used *mdx:utr^{-/-}* mice, a severe mouse model of DMD that exhibits a pronounced cardiomyopathic phenotype. We provide the first direct evidence implicating ferroptosis in the pathology of DMD-CM associated with dystrophin and utrophin deficiency, as demonstrated by Live/Dead cell viability assays. These findings suggest that DMD renders cardiomyocytes more susceptible to ferroptosis under stress conditions. Moreover, using *mdx:utr^{-/-}:sln^{+/-}* mice, we found that SLN reduction alleviates ferroptosis susceptibility in DMD-CM, which is consistent with our previous findings that lowering SLN levels mitigates skeletal muscle and cardiac pathology in DMD and may have clinical implications [9]. These results indicate that ferroptosis-mediated cardiomyocyte loss is, at least in part, involved in the pathogenesis of DMD-CM and contributes to progressive cardiac dysfunction and eventual heart failure. Elucidating the link between ferroptosis and DMD-CM may therefore uncover novel therapeutic targets for DMD-CM.

4.2. Ferroptosis-Related Biomarkers and Ferroptotic Pathways in the Context of DMD-CM

Thus far, no specific assay is available for the selective staining of ferroptotic cells in tissue sections [28]. Instead, molecules that serve as either drivers or consequences of ferroptosis have been employed as ferroptosis-related biomarkers, which may then be applied to its detection and support the diagnosis, prognosis, and treatment of ferroptosis-associated diseases [41–44]. These ferroptosis-related biomarkers include elevated levels of redox-active iron, excessive lipid peroxide accumulation, and alterations in key genes and proteins that govern iron homeostasis, lipid metabolism, and ferroptotic signaling pathways.

In our present study in *mdx:utr^{-/-}* mouse hearts (a model of DMD-CM), characteristic features of ferroptosis, including increased iron availability and lipid peroxide accumulation, were evident at the tissue level (Figure 1A,B). We further evaluated ferroptosis-related biomarkers and identified key pathways that may play critical roles in ferroptosis in the setting of DMD-CM. We found upregulation of TfR1, 15LOX1, COX2, and FACL4 in *mdx:utr^{-/-}* mouse hearts (Figure 5A,B), consistent with their roles as drivers of ferroptosis. A marked tendency toward increased expression of TfR1, 15LOX1, and FACL4 was also observed in heart tissues of DMD patients. TfR1 is the predominant transferrin receptor expressed in cardiac cardiomyocytes. It plays a crucial role in iron uptake by mediating the endocytosis of iron-bound transferrin, which is essential for maintaining iron homeostasis in the heart. Given the high metabolic demands of cardiomyocytes, iron is necessary for

mitochondrial function and energy production. In the context of the occurrence of ferroptosis in cardiovascular diseases, including the DMD-CM, TfR1 seems to be particularly relevant. In our present study, TfR1 protein expression (Figure 5 and Supplement Figure S2) was consistently upregulated in the heart tissues of DMD mice and DMD human patients. Among the so-called ferroptosis-related biomarkers, TfR1 is currently considered the most convincing and specific indicator of ferroptosis [41]. This is because: (1) it directly links to cell iron uptake and metabolism driving lipid peroxidation, which is hallmark of ferroptosis; (2) TfR1 expression is specifically elevated under ferroptotic stress, but not in other forms of regulated cell death; (3) it is possible that TfR1 antibodies can be used to detect ferroptotic cells in tissues, making it a practical histological and biochemical marker in cell culture and tissue-based experimental contexts. Therefore, a combination of anti-TfR1 and anti-MDA antibodies has been proposed to detect ferroptotic cells in diverse contexts [41].

We also found the downregulation of FSP1 consistent with its important role as a ferroptosis suppressor. Recent studies have shown that HMOX1 [26,45–48] and NOX4 [49–52] may play either a pro-ferroptosis role or an anti-ferroptosis role under different conditions (e.g., disease models, cell types). Specifically, HMOX1 can have either a protective or deleterious effect on cardiac function depending on its activation level and basal oxidation conditions [53]. Our data have shown the downregulation of both HMOX1 and NOX4 in *mdx:utr^{-/-}* mouse hearts, suggesting they may serve as ferroptosis suppressors in the setting of DMD-CM. This is consistent with the report that the expression of HMOX1 is blunted in the skeletal muscles from the *mdx* mouse model and overexpression of HMOX1 reverses the DMD phenotype [35].

GPX4 is a well-recognized key suppressor of ferroptosis and functions to detoxify lipid peroxides in various cell types [18,25,54]. GPX4 specifically targets phospholipid hydroperoxides within membranes, thereby making it uniquely important for maintaining membrane integrity. Its inhibition/downregulation leads to unchecked lipid peroxidation and ferroptosis. For example, direct inhibition of GPX4 (e.g., by RSL3) is a potent trigger for ferroptosis. Nevertheless, we observed no difference in GPX4 expression in *mdx:utr^{-/-}* mouse hearts compared to WT, suggesting that cardiomyocyte ferroptosis in DMD-CM may be governed less by GPX4 protein abundance. Several factors may account for this: (1) GPX4 activity may be regulated at the enzymatic or cofactor level (e.g., GSH availability, selenocysteine incorporation) rather than by expression changes; (2) ferroptosis susceptibility can be modulated via GPX4-independent pathways, such as the FSP1–CoQ10 axis or mitochondrial DHODH [55]. Tissue or context-specific mechanisms, including iron overload, lipid components, or Ca^{2+} dysregulation, may render GPX4 less of a rate-limiting factor than other ferroptosis drivers (e.g., FACL4, TfR1, 15LOX1). The potential biomarkers and pathways most likely involved in ferroptosis in DMD-CM are depicted in Figure 6.

In our earlier study, we have shown that SLN expression is upregulated in *mdx:utr^{-/-}* mouse hearts that manifest CM pathology, while reducing SLN expression (*mdx:utr^{-/-}:sln^{+/-}*) ameliorates dystrophic cardiac pathology in DMD-CM [9]. Our present study demonstrates that reducing SLN expression (*sln^{+/-}*) mitigates the enhanced ferroptotic susceptibility of *mdx:utr^{-/-}* cardiomyocytes, in agreement with previous findings on heart function and lifespan [9]. However, the expression levels of ferroptosis-related biomarkers in *mdx:utr^{-/-}:sln^{+/-}* hearts were not significantly reversed from hearts in which there was no reduction in SLN expression (i.e., *mdx:utr^{-/-}*) (Figure 5). This suggests that other mechanisms or pathways may also be involved. For example, sarcolemmal instability under DMD conditions leads to Ca^{2+} influx, thereby exacerbating mitochondrial dysfunction and increasing ROS generation. It has been suggested the interplay between Ca^{2+} dysregulation/overload and ferroptosis may amplify cardiomyocyte damage [56].

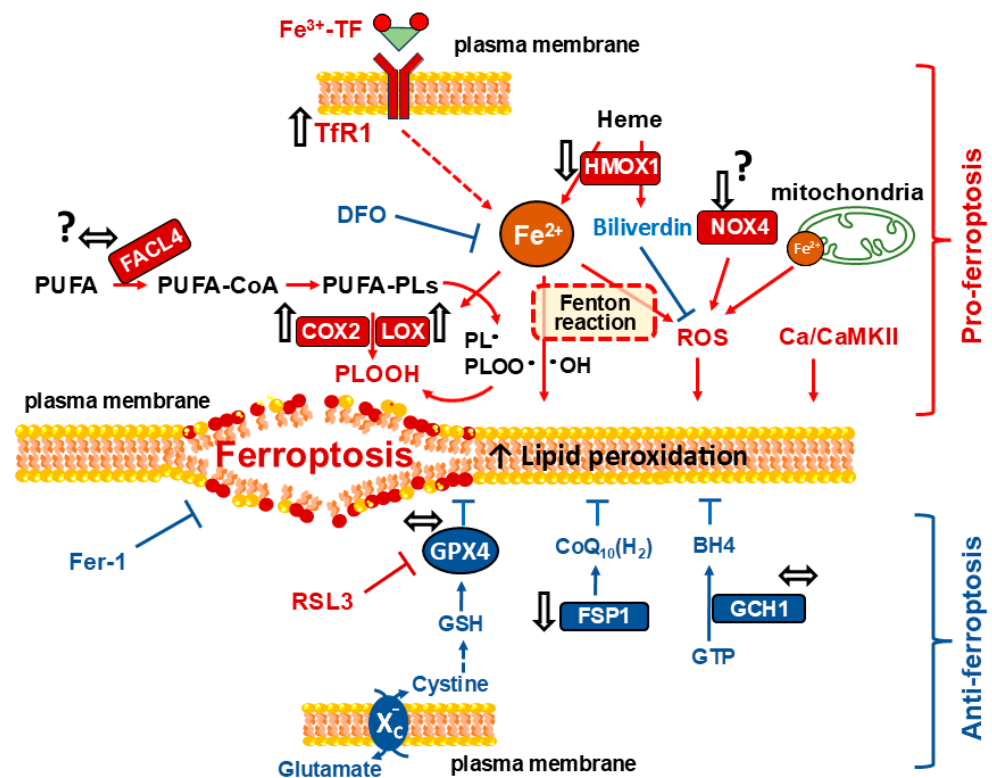


Figure 6. A schematic illustration depicting putative alterations in molecular pathways that contribute to the increased susceptibility to ferroptosis in DMD-CM. Pro-ferroptosis (red) and anti-ferroptosis (blue) pathways are shown. Upregulation and downregulation of the molecules are represented by upward and downward hollow arrows, respectively. Question marks indicate uncertainty in the regulatory roles. BH4: tetrahydrobiopterin; CaMKII: Calcium/calmodulin-dependent protein kinase II; CoA: coenzyme A; CoQ10: coenzyme Q10; DFO: deferoxamine; FACL4: Fatty acid-CoA ligase 4; Fer-1: ferrostatin-1; FSP1: ferroptosis suppressor protein 1; GPX4: glutathione peroxidase 4; GSH: glutathione; GCH1: GTP cyclohydrolase 1; HMOX1: heme oxygenase 1; LOX: lipoxygenase; NOX4: NADPH oxidases 4; PL•: phospholipid radical; PLOO•: phospholipid peroxy radical; PLOOH: phospholipid hydroperoxides; PUFA: polyunsaturated fatty acid; ROS: reactive oxygen species; RSL3: RAS-selective lethal 3; TfR1: Transferrin Receptor 1; COX2: Cyclooxygenase-2.

4.3. Study Limitations

This study has certain limitations that should be acknowledged. These findings suggest potential clinical relevance by evaluating ferroptosis-related biomarkers in human DMD patient heart tissues. However, the number of samples analyzed was limited (e.g., only two human heart samples in each group), which precluded statistical significance. The findings from this study are preliminary and need to be validated in larger samples to establish their clinical significance. Additionally, the DMD human samples lack dystrophin but retain utrophin expression, which should be taken into account when comparing human and mouse data as the list of ferroptosis-related biomarkers continues to expand [57]. We examined a subset of biomarkers and pathways, leaving other potentially important candidates unexamined. Although significant associations were observed, the underlying mechanisms remain to be elucidated.

Fer-1 is a radical-trapping antioxidant that suppresses lipid peroxidation and is widely used as a selective inhibitor of ferroptosis. Despite this being well known, we appreciate that Fer-1 treatment alone cannot fully exclude the involvement of other forms of regulated cell death. Therefore, in addition to ferroptosis, we cannot exclude the contribution of other cell death types (e.g., necroptosis) to the development of DMD-CM pathology. Nevertheless, FAC and RSL3 have been well used to induce ferroptosis in cardiomyocytes [22,23], which

shows iron dependence and/or selective sensitivity to the ferroptosis inhibitor Fer-1, but not to the apoptosis inhibitor EMR or necroptosis inhibitor necrostatin-1 [22]. Although our sample size (n) was limited due to the scarcity of *mdx:utr*^{−/−} mice, the data shown in Supplement Figure S1 indicate that the cell death in *mdx:utr*^{−/−} cardiomyocytes was iron-dependent and was insensitive to inhibition of apoptosis. Therefore, it is reasonable to conclude that the ferroptosis susceptibility is increased in the setting of DMD-CM.

Despite these limitations, this study, using *mdx:utr*^{−/−} mouse model, provides valuable insights into DMD-CM pathology and, for the first time, demonstrates the importance of ferroptosis in DMD-CM. Further detailed studies are warranted to demonstrate the mechanistic links and potential clinical relevance of the current study. Newer evidence suggests that D2 *mdx* mice (*mdx* mutation on a DBA/2J background) represent a more clinically relevant DMD model [58–61] and may be preferable for future studies. Therefore, we plan to utilize the D2 *mdx* model in the future to validate our present observations. The current data obtained using the *mdx:utr*^{−/−} mouse model, however, is the first to demonstrate the importance of ferroptosis in DMD-CM.

4.4. Conclusions

Progressive loss of cardiomyocytes and the resulting cardiac remodeling lead to dilated cardiomyopathy and heart failure in DMD patients. Understanding the mechanisms of cell death is critical for developing targeted therapies to preserve cardiac function. Our present study provides the first direct evidence suggesting a strong association between ferroptosis and the pathogenesis of DMD-CM. Furthermore, reducing SLN levels mitigates ferroptosis and has clinical implications in the treatment of DMD-CM. These results are consistent with previous functional studies on both skeletal muscle and hearts [9].

Current management of DMD-CM relies on standard heart-failure therapies, including ACE inhibitors, ARBs, β -blockers, glucocorticoids, and mineralocorticoid receptor antagonists, which slow disease progression but do not address the underlying molecular pathology. Emerging strategies target calcium dysregulation, oxidative stress, inflammation, and mitochondrial dysfunction, while gene-based therapies such as exon skipping and micro-dystrophin delivery hold promise for restoring dystrophin and modifying the disease course [6]. Despite these advances, effective cardiac-specific therapies remain limited, highlighting the need for novel, mechanism-based interventions.

Our present study provides evidence that ferroptosis may contribute to the pathogenesis of DMD-CM. Thus, we speculate that inhibiting ferroptosis with specific inhibitors or by downregulating SLN could potentially complement current therapeutic approaches for DMD, although the clinical translation of gene-based strategies remains challenging. Furthermore, in addition to ferroptosis, other types of cell death should also be considered.

In conclusion, ferroptosis likely plays a crucial role in the pathogenesis of DMD-CM, and suppressing cardiac ferroptosis is expected to become a promising therapeutic option. Iron chelators, antioxidants, ferroptosis inhibitors, and genetic manipulations may alleviate DMD-CM by blocking ferroptosis pathways. Future studies are warranted to assess the therapeutic potential of targeting ferroptosis pathways, including small-molecule ferroptosis inhibitors, iron chelation, antioxidants, and SLN reduction, in both animal models and human patients with DMD-CM.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/biomedicines14020472/s1>, Figure S1. Incidence of ferroptosis in *mdx:utr*^{−/−} cardiomyocytes in the absence (Ctl) and presence of the apoptosis blocker emricasan (EMR) or the iron chelator deferoxamine (DFO). Figure S2. Assessment of ferroptosis-related molecules in heart tissues from DMD patients compared to those of non-DMD controls.

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Informed Consent Statement: The IRB determined that this study was exempt. The study used existing de-identified specimens. The samples were obtained from the University of Maryland Brain and Tissue Bank, a member of the NIH NeuroBioBank network. No informed consent forms were held by the investigators.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

Abbreviations

15LOX1	15 lipoxygenase-1 or Arachidonate 15 lipoxygenase
ACSL4	acyl-CoA synthetase long-chain family member 4 (AKA. FACL4: Fatty acid-CoA ligase 4)
BH4	tetrahydrobiopterin
CaMKII	calcium/calmodulin-dependent protein kinase II
CoA	coenzyme A
CoQ10	coenzyme Q10
COX2	cyclooxygenase-2
Fer-1	ferrostatin-1
FSP1	ferroptosis suppressor protein 1
DFO	deferoxamine
EMR	emricasan
GPX4	glutathione peroxidase 4
GSH	glutathione
GCH1	GTP cyclohydrolase 1
HMOX1	heme oxygenase 1
hiPSC-CM	human iPSC-derived ventricular cardiomyocytes
LOX	lipoxygenase
NOX	NADPH oxidases
PL•	phospholipid radical
PLOO•	phospholipid peroxy radical
PLOOH	phospholipid hydroperoxides
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
RSL3	RAS-selective lethal 3
TfR1	transferrin Receptor

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